Mammalian MutS Homolog 5 is Required for Chromosome Pairing in Meiosis

Winfried Edelmann[1], Paula Cohen[2], Burkhard Kneitz[1], Nina Winand[3,4], Joerg Heyer[5], Richard Kolodner[3,6], Jeffrey Pollard[2] and Raju Kucherlapati[5]

[1]Department of Cell Biology, [2]Department of Developmental and Molecular Biology, [5]Department of Molecular Genetics, 1300 Morris Park Avenue, Bronx, NY 10461, Albert Einstein College of Medicine, Bronx, NY, [3]Dana Farber Cancer Center, 44 Binney Street, Boston, MA, [4]Present Address: Cornell University, Ithaca, NY, [6]Present Address: Ludwig Cancer Institute, University of California, San Diego, La Jolla, CA

Address correspondence to: Winfried Edelmann
Department of Cell Biology
Albert Einstein College of Medicine
1300 Morris Park Avenue
Bronx, NY 10461
edelmann@aecom.yu.edu

MutS homolog 5 (MSH5) is a member of a family of proteins that are known to be involved in DNA mismatch repair. Unlike some other members of this family, which are ubiquitously expressed, MSH5 expression is restricted with high expression in gonadal tissues. To assess the role of MSH5, we developed mice carrying a null mutation in the MSH5 gene. Mice homozygous for this mutation are viable but are sterile. In males the sterility is the result of the failure of pairing of homologous chromosomes although they do associate with proteins of the synaptonemal complex and Rad51, a marker for early recombination nodules. This results in the complete loss of post-zygotene spermatocytes, no sperm and a 70% reduction of testis size. In females, oocytes are present up to 3 days post-partum but are rapidly depleted with a complete loss by day 6 post-partum. The ovaries are of normal size at birth, but degenerate and are entirely absent in the adult. The time of meiotic blockage in MSH5 mutant mice implicates this protein in a completely novel function of initiation of homologous pairing in meiosis. Our results also show that meiotic synaptic failure in embryonic ovaries leads to a failure in developmental remodeling of the ovary, loss of the oocyte pool and complete ovarian degeneration in early adulthood, providing an explanation of the ovarian phenotype in Tuner syndrome patients.

Mismatched bases in DNA result from a number of mechanisms including errors in DNA replication, genetic recombination and modification of DNA or nucleotides. The enzymatic mechanisms that are capable of repairing mismatched nucleotides in DNA are reletively well conserved in prokaryotes and eukaryotes. The bacterial DNA mismatch repair systems typified by those found in E. coli are among the simplest and best understood. Among the systems that repair mismatches, the MutHLS system has been most extensively studied. This system is capable of repairing both single nucleotide mismatches as well as small insertion/deletion mismatches (for reviews, see Modrich 1991; Kolodner et al., 1995, 1996; Modrich and Lahue, 1996). In this system, the MutS protein binds to DNA at the site of the mismatch and is required for mismatch recognition. A second protein, MutL, interacts with MutS when MutS is bound to a mispaired base and this ternery complex plays a crucial role in the activity of the proteins that are responsible for the excision and resynthesis steps of mismatch repair.

A remarkable observation made in eukaryotes, initially in yeast and later in humans, is that MutS and MutL related proteins that are essential components of the mismatch repair system have been conserved between eukaryotes and prokaryotes. The repair system is, however, more complex and involves several MutS and MutL homologs. In the yeast Saccharomyces cerevisiae there are six homologs of the mispair recognition component of the reaction, MutS (Reenan and Kolodner, 1992a, 1992b; New et al., 1993; Ross-McDonald and Roeder, 1994; Hollingsworth et al., 1995; Marsischky et

al., 1996) designated MutS homolog (MSH) 1-6. There are four known homologs of the MutL gene in yeast, designated MLH1-3 and PMS1 **(for a review see Crouse, 1998)**. The mammalian genome encodes homologs for most of these genes including 5 of the 6 MutS homologs, MSH2-6. The products of MSH2, MSH3, MSH6, as well as MLH1 and PMS1 (PMS2 in humans) are clearly involved in DNA mismatch repair. However, in contrast to bacterial mismatch repair were a single MutS protein is required, eukaryotic mismatch repair utilizes MSH2-MSH3 and MSH2-MSH6 complexs to recognize different types of **mismatches** in DNA and an MLH1-PMS1 complex that interacts with the MSH2 complex instead of a single MutL protein **(Kolodner et al., 1995, 1996; Modrich and Lahue, 1996)**.

In S. cerevisiae, there is considerable evidence that MutS and MutL related proteins are required for normal meiosis. The roles of these proteins in meiosis include their requirement for the repair of mispaired bases in meiotic recombination intermediates as well as apparantly a role in promoting crossing over that is distinct from a role in mismatch repair (**Petes** et al., 1991; Reenan and Kolodner, 1992; Prolla et al., 1994; Ross-McDonald and Roeder, 1994; Hollingsworth et al., 1995; Williamson et al., 1985; **Alani et al, 1994**). One impetus for the study of mismatch repair genes in humans came from the identification of the roles of MSH2 and MLH1 in a cancer predisposition syndrome, hereditary non-polyposis colon cancer (HNPCC) (Lynch syndrome) as well as in the development of some types of sporadic cancers. However, it is also important to understand the roles of these genes in meiosis since many of the

meiotic defects seen in mutant S. cerevisiae strains resemble the types of meiotic defects seen in some types of infertility in humans.

To understand the role of the mismatch repair genes in DNA repair, cancer predisposition and meiosis, we and several other investigators have begun to generate mice by gene targeting that are mutant in each of the genes encoding MutS and MutL homologs and examine their phenotypes. Mice which carry mutations in Msh2 (deWind et al., 1995; Reitmair et al., 1995; Fodde et al., unpublished observations), mlh1 (Edelmann et al., 1996; Baker et al., 1996), PMS2 (Baker et al., 1995), PMS1 (Reference) and Msh6 (Edelmann et al., 1997) have been described. Although all of the genes examined to date encode proteins that function in the same complex involved in recognition of mismatches in DNA, mice lacking different genes have some common and some distinct features. Qualitatively, Msh2 -/-, Mlh1 -/-, Msh6 -/- and PMS2 -/- show a disposition to develop tumors, although the degree of this predisposition and the time after birth when tumors develop differ. Mice lacking PMS1 appear to be normal in this regards.

A remarkable observation made during the studies of the mutant mice is that some of them have meiotic defects while others do not. Mice with mutations in the MSH gene family (MSH2 and MSH6), in the homozygous state, are viable and fully fertile (deWind et al., 1995; Reitmair et al., 1995; Edelmann et al., 1997). Mice with a homozygous mutation in PMS2 show abnormal chromosome pairing during male meiosis and are male sterile whereas the females are fertile (Baker et al., 1995). Mice with mutations in the Mlh1 gene are viable but both sexes are sterile. Normal

chromosome pairing was observed in pachytene but most of the cells fail to progress beyond pachytene and seem to be shunted into an apoptotic pathway. A few cells that survive this shunt show premature separation of homologous chromosomes.

How could inactivation of genes (MSH2, MSH6 and MLH1) whose products are known to interact yield related repair defects and cancer predisposition phenotypes, cause extremely diverse meiotic phenotypes? A possible explanation is that MLH1, MSH2 and MSH6 interact and are members of a complex involved in repair of DNA mismatches in somatic cells but that MLH1 employs a different member of the MSH family as its partner in meiosis. The fact that in yeast mutations in MSH4 and MSH5 cause a meiotic phenotype (Ross-McDonald and Roeder, 1994; Hollingsworth et al, 1995) and that these genes are expressed in germ cells in mammals (Paquis et al., 1997; Winand et al., 1998) suggested that one or both of these gene products may be the partners for Mlh1.

To ascertain the role of MSH4 and MSH5 in mammals, we initiated an effort to generate mice with mutations in each of these genes. We now report the generation and characterization of mice with a null mutation in Msh5. Msh5 -/- mice are viable but males and females are sterile. Meiosis in these mice is severely affected where the chromosomes fail to pair. This meiotic failure leads to a rapid and progressive attrition of testes and ovaries. Our results show that normal MSH5 function is essential for meiotic progression and gonadal maintenance.

RESULTS

Isolation of mouse cDNA and genomic clones for MSH5. The isolation of human **MSH5 cDNA, its genomic** organization and expression patterns were described **(Winand** et al., 1998; Doggett et al., 1998). **As part of the present study, 2.7 kb of mouse MSH5 cDNA sequence was defined by sequencing 5'- and 3'-RACE products** (Figure 1). **This mouse cDNA contains an open reading frame corresponding to 834 a.a. The coding region of the mouse cDNA shares 86% sequence identity and the predicted amino acid sequence shares 89% identity with the human cDNA and predicted amino acid sequence, respectively.** The high level of conservation in the DNA sequence and the deduced amino acid sequence suggest functional conservation in the two species. **In addition, we determined the sequence of introns 10, 17, 18, 19 and 21 by sequencing selected regions of P1 clones containing the mouse MSH5 gene and found that the mouse and human genes ahd the same structural organization in these regions (data not shown).**

Generation of mice with a disrupted Msh5 gene. A 344 bp PstI fragment of the mouse cDNA which contains exon 16 of the gene was used to screen a mouse genomic λ phage library derived from the strain 129/Ola. From one of the positive clones, we isolated a 3.8 Kb HindIII fragment that contained exons 15 and 16 of the Msh5 gene. The gene targeting scheme that we used to generate mouse embryonic stem (ES) cells is shown in Figure 2. **The** gene targeting vector designated pMSsh5ex16 was constructed by insertion of a PGK-hygromycin gene cassette into the unique Aat II site in exon 16 of the gene. The hygromycin expression cassette is the opposite transcriptional orientation to

that of Msh5. As a result, any transcript that is derived from the modified gene would contain a number of in-frame stop codons. pMsh5ex16 was linearized and introduced into ww6 ES cells. One hundred and forty-four of the hygromycin resistant clones were screened by PCR for the desired gene modification event. Six of the 144 (4%) had the desired modification. Two of these cell lines, MSH5-1 and MSH5-52 were injected into blastocysts derived from C57BL/6 females. Both cell lines yielded robust chimeras which transmitted the modified locus through their germ line.

Homozygous Msh5 -/- mice are viable. To examine the effect of the Msh5 gene mutation on the viability of mice, we interbred Msh5 +/- mice. The offspring from several such matings were genotyped by PCR and in some cases by Southern blotting. For Southern blotting, tail DNA was digested with NsiI and hybridized with a probe located outside the targeted region. Representatative results are shown in Figure 2. The wild-type locus is represented by a 2.0 Kb fragment while the modified locus is represented by a 4.0 Kb band. We obtained three classes of mice corresponding to +/+, +/- and -/- genotypes. Of 606 mice that were examined, 184 were +/+, 275 were +/- and 147 were -/-. These results show that mice which have a disrupted Msh5 gene in the homozygous state are viable.

Msh5 -/- mice do not express Msh5 transcripts or protein. To examine the expression of the modified Msh5 locus, we prepared RNA from testes of 24 day old Msh 5 +/+ and -/- mice and did Northern blot analysis with different probes. The results are shown in Figure 3. When an Msh5 probe corresponding to exons 3-8 of the gene was used, RNA from +/+ mice gave a 2.0 Kb band. No such band was detected in RNA

from testes of -/- mice. To ensure that testes specific RNA was indeed used, we hybridized the same blots with a probe corresponding to Msh4 and §-actin. Testes RNA from both +/- and -/- mice contained equivalent amounts of §-actin. Both samples also were positive for Msh4 transcripts, although their levels are lower in the mutant homozygotes. We believe that the reduced Msh4 transcripts reflect a reduction in germ cells in 24 days old males (see below).

We also examined for the presence of MSH5 protein in testes. Protein extracts from testes of 24 days old males were tested by Western blot analysis using a COOH and specific antibody (Figure 3). The antibody reacted with the expected 93 Kd MSH5 protein in extracts from +/+ and +/- mice but no such band was detectable in extracts from -/- mice. Taken together, the Northern and Western blot data suggest that the modified Msh5 locus does not produce a functional MSH5 protein.

Msh5 -/- males are sterile. The restricted localization of *msh5* to the gonads of mice and humans (Fig. X; Winand et al, 1998), together with the meiotic phenotype of *msh5* yeast mutants, suggests a role for *msh5* in mammalian meiosis. Detailed analysis of msh5 mRNA expression in prepubertal testis is consistent with this role. The onset of *msh5* expression is co-incident with the onset of the first wave of meiosis in the mouse testis, beginning at day 11 postpartum (pp) and peaking at day 23-26 pp (Fig. 4A). This temporal expression pattern closely mirrors the acquisition of early spermatocytes in the seminiferous tubules and peaks at a time when the proportion of cells in prophase I is maximal, suggesting that any role of msh5 in meiosis must be limited to early meiosis. Therefore, we analysed spermatogenesis and male sexual behaviour in msh5-

deficient mice. Homozygous mutant males demonstrated normal sexual behavior in the presence of wildtype females, and their mating frequency, as assessed by the formation of vaginal plugs in receptive females, was identical to that of +/+ and +/- males. However, such matings never led to pregnancies. Examintation of the testes of these *msh5* -/- males showed that they were 70% smaller than age-matched controls and, in the epididymides there was a complete lack of spermatozoa while wildtype and heterozygous mice had epididymal sperm numbers within the normal range (mean of 4.72×10^6 sperm per mouse).

Examination of seminiferous tubule cytology in adult males demonstrated a severe disruption of spermatogenesis in *msh5* -/- males compared to the normal abundance of all stages of the spermatogenic wave in wildtype males (Fig. 4B,C) and this was the cause of the reduction in testis size and absence of epididymal spermatozoa. In the mutant adult testis, interstitial Leydig cells and tubular Sertoli cells are present, as are type A and B spermatogonia (Fig. 4D,E) but, in contrast to wildtype testes (Fig. 4D), no spermatocytes are observed beyond zygotene (Fig. 4E). Immunohistochemical staining with anti-germ cell nuclear antigen 1 (GCNA1) revealed positive cells in the *msh5* -/- testis confirming the presence of germ cells whose position and size are indicative of early type A and B spermatogonia and pre-pachytene spermatocytes (Fig. 4G). In contrast, wild type males display a complete complement of spermatogenic cells including cells at pachytene, metaphase I, meiosis II, differentiating spermatids and fully differentiated spermatozoa (Fig. 4F).

In mice, the onset of prophase I of the first wave of spermatogenenesis commences at day 13 pp and is complete by day 25 pp, allowing the identification of discrete stages of meiotic progression. At day 17 pp, the seminiferous epithelium of wildtype males is densly packed with proliferating germ cells and with spermatocytes up to mid-pachytene (Fig. 5A; Bellve, 1977). In *msh5* -/- males, the seminisferous tubules are also fairly densly packed, although early signs of germ cell loss are evident, both by reduced GCNA1 positive cells and by the increase in apoptotic (TUNEL-positive) cells relative to that seen in wildtype tubules (Fig. 5B,C,D). By day 23 pp, the tubules of wildtype mice have progressed through an entire meiotic cycle and round spermatids have become evident by the loss of GCNA1 signal (Fig. 5E). In constrast, the tubules of *msh5* -/- males have lost significant mubers of germ cells (Fig. 5F) due to the sustained elevated level of apoptosis relative to that in wildtype seminiferous tubules (Fig. 5G,H). Thus, by 6 weeks of age, wildtype testes contain seminferous tubules packed with all stages of spermatogenesis (Fig. 5I) while the seminifours tubules of *msh5* -/- males have lost most of their cellular components beyond the level of the spermatogonia (Fig. 5J). The majority of remaining spermatocytes in the adult *msh5* -/- males are undergoing apoptosis (Fig. 5L). Thus, in the absence of *msh5*, there is a rapid and depletion of meiotic cells during the first wave of meiosis. Cells continue to be lost from the seminiferous tubules of *msh5* -/- males with each successive spermatogenic wave such that, by adulthood, almost the eintire spermogenic cell population is lost.

Meiotic arrest in *msh5* -/- males. The loss of spermatocytes from the first spermatogenic wave onwards in *msh5* -/- males suggests that there is a failure of

meiosis during early prophase I in these mice. Therefore, in order to analyse meiotic progession in further detail, meiotic chromosome spreads were prepared and studied at the light and electron microscope level. Chromosomes were prepared from testicular cell suspensions of 23 day old males to minimize the numbers of spermatids and spermatozoa present and to maximize the numbers of spermatocytes retrieved. In wildtype spreads, silver staining revealed a range of chromosomal configurations, including those at leptotene, zygotene, pachytene and diplotene. Chromosomes at leptotene and zygotene were unpaired, but at the latter stages of zygotene were beginning to condense and showed early signs of pairing. By pachytene, homologous chromosomes were synapsed along their entire lengths and were more fully condensed (Fig. 6A). In contrast, chromosome preparations from *msh5* -/- testes at day 23 pp did not contain any synapsed chromosomes although the chromosomes appeared to have replicated **to produce** sister chromatids (Fig. 6B), and all the spermatocytes examined appeared to be at the leptotene/zygotene stages of meiosis with only minimal condensation of the univalent chromosomes (Fig. 6B).

In order to determine whether the meiotic program is initiated in spermatocytes from *msh5* -/- males, we examined the chromosomal association of three proteins known to be required for recombination and formation of the synaptonemal complex (SC). The first two, SYCP1 and SYCP3, form integral components of the SC. SYCP1 (detected using the anti-syn1 antibody) is an integral component of the transverse filaments of the SC and is expressed from the zygotene to diplotene stages of prophase 1. SYCP3 (detected using the anti-cor1 antibody) is present on unpaired chromosome

axial cores prior to synapsis and is expressed from leptotene stages onwards. Immunofluorescant localization of these proteins on meiotic chromosomes using a combined antisera demonstrated normal acquisition of SC in spermatocytes from wildtype males and identified pachytene spermatocytes as having 20 distinct condensed pairs of bivalents (Fig. 6C). In *msh5* -/- spermatocytes, however, no bivalents were observed, but univalent chromosomes were clearly associated with the SYCP1/SYCP3 signal, indicating that axial element formation has been achieved and suggesting progression of meiosis at least through until mid-zygotene (Fig. 6D).

Rad51, a mammalian homolog of the bacterial RecA protein, is thought to participate in heteroduplex formation and/or chromosome repair following double strand break formation, and is an integral component of recombination nodules in lilies and possibly in mice. In wildtype spermatocytes, discrete Rad51 foci are located along the entire length of chromosomes during leptotene (Fig. 6E) and decrease steadily from zygotene through to pachytene as chromosomes become fully paired. In msh5 -/- spermatocytes, Rad51 is also localized in discrete foci along the univalent chromosomes (Fig. 6F) and, indeed, the number and intensity of these foci appears greater in the majority of msh5 -/- cells than on leptotene or zygotene chromosomes from wildtype males. Thus, the localization of Rad51 foci on the meiotic chromosomes from msh5 -/- spermatocytes does not decline, concurrent with the failure of synapsis in these cells.

Msh5 -/- females show a loss of oocytes and ovarian degeneration throughout life. To examine the role of **Msh5** in female meiosis, ovarian function was assessed in msh5 -/- adults. Msh5 -/- adult females did not mate with wildtype males, nor did they exhibit

characteristic estrous cycles. Gross anatomical observations indicated that the msh5 -/- females have normal oviducts, normally structured but hypotrophic uteri but lack discernible ovarian structures (Fig. 7D,E). Instead, the ovarian bursa of msh5 -/- females was empty or more frequently contained only a single cyst-like structure or a grouping of 3-4 cysts (Fig. 7E). To assess whether the absence of ovarian structure was due to developmental impairment or due to post-developmental degeneration of the ovary, ovaries from wildtype and msh5 -/- females were examined at day 3 pp and day 25 pp. At day 3 pp, there was no difference in ovarian size between wildtype and msh5 -/- females (Fig. 7A,B), although the ovaries of msh5 -/- females contained apparently fewer oocytes, as assessed by GCNA1 localization using an anti-GCNA antibody (Fig. 7B). By day 25 pp, the ovaries of msh5 -/- females were reduced to a small grouping of 1-3 follicles which appeared to be at post-antral stages of development, and occasionally contained oocytes (Fig. 7C). Wildtype ovaries at the same stage are similar to adult ovaries but with fewer corpora lutea and an abundance of primordial follicles (not shown). The presence of oocytes in the day 25 pp msh5 -/- females was confirmed by RT-PCR detection of transcripts for the oocyte-specific protein, zona pellucida 3 (ZP3) at day 25 pp in both wildtype and homozygous mutant females, However, ZP3 transcripts could only be detected in wildtype ovaries at adulthood (Fig. 7F). Thus, the ovaries of msh5 -/- females are normal size at birth, but then degenerate progressively to become rudimentary by adulthood. This ovarian degeneration is concomitant with a decline in oocyte numbers from before day 3 pp until adulthood, when no oocytes are present.

Msh5 -/- oogonia are meiotically incompetent and are almost entirely lost within the first 6 days of life. To examine the cause of the lower oocyte numbers in msh5 -/- females on day 3 pp, ovarian histogenesis was analyzed in female embryos from heterozygote mothers. Embryonic ovaries were removed from wildtype and *msh5* -/- females between embryonic day 15 (e15) and day 6pp. Alkaline phosphatase staining of e15 ovaries revealed normal acquisition of germ cells into the genital ridge in both female and male embryos (data not shown). Pre-meiotic and meiotic oocytes were then tracked using the anti-GCNA1 antisera between e16 and d6pp. At the early stages (e18 until day 1pp), the ovaries of homozygous mutant females contain apparently normal numbers of oocytes compared to wildtype (Fig. 8A,C). However, closer examination of H&E stained sections revealed subtle differences in chromosome structure between wildtype and msh5 -/- oocytes, characterized by clumping of nuclear contents in the homozygous mutant oocytes (Fig. 8D) compared to readily identifiable chromosomes in the wildtype oocytes (Fig. 8B). By day 3 pp, the number of oocytes present in the ovaries of msh5 -/- females was dramatically lower than that in wildtype ovaries (Fig. 8E,G), with more distinct morphological differences being notable; many oocytes from wildtype mice had grown considerably in size and were beginning to become encapsulated within primordial follicles, at the same time losing their anti-GCNA1 staining (Fig. 8F). In contrast, the ovaries of homozygous mutant females fail to undergo the expected architectural changes characteristic of early folliculogenesis, with oocytes remaining small and their nuclei retaining their dense staining with anti-GCNA1 antibody (Fig. 8H). By day 6 pp, large, well-formed primordial follicles

containing readily identifiable oocytes were distributed throughout the ovary of wildtype females (Fig. 8I, J), while in ovaries from msh5 -/- females the oocyte pool was completely diminished (Fig. 8K,L), with few or no GCNA1-positive oocytes being evident. Thus, in the absence of msh5, the ovaries become rapidly depleted of oocytes within days of the initiation of meiosis. Interestingly, the somatic tissues of the ovary fails to reorganize and to initiate folliculogenesis on day 2 pp as expected, despite the fact that the oocytes are not lost until after day 3 pp and this leads to the progressive loss of ovarian structure as described above.

DISCUSSION

Eukaryotes have a number of genes which are homologous to MutS. Of these, MSH2, MSH3 and MSH6 are ubiquitously expressed and form MSH2-MSH3 or MSH2-MSH6 complexes which are important for recognition of mispaired bases during mismatch repair. In yeast, MSH4 and MSH5 have distinct functions because mutations in these genes do not seem to affect mismatch repair but do affect meiosis resulting in reduced crossing over and reduced spore viability consistent with a defect in chromosome segregation. In mice and humans, MSH4 and MSH5 genes have more limited patterns of expression with maximal expression of MSH5 being found in gonads and lymphoid tissues. To assess the role of MSH5, we generated mice which have a mutation in this gene. There are two lines of evidence that suggest that the mutation is a null. We have introduced a hygromycin resistance cassette into exon 16 of the MSH5 gene disrupting an exon common to all alternatively spliced MSH5 transcripts. If transcripts are made from this modified locus, the translated product will not contain

the nucleotide binding domain and the helix-loop-helix domain located at the COOH-end that are important for the function of this family of proteins. Northern blot analysis of testis RNA from MSH5 -/- mice showed no MSH5 RNA. Our ability to detect MSH4 transcripts in these blots provided confidence that the RNA is derived from the appropriate source. Western-blot analysis clearly showed that the COOH end of the protein is absent. It is of interest to note that the levels of MSH4 RNA are reduced in testicular RNA. This could be explained by the depletion of germ cells.

The most striking phenotype in MSH5 deficient mice is that they are sterile consistent with **some** type of meiotic defect. This phenotype was detected in both sexes. The mice appear normal and males mate normally with wild-type females with vaginal plugs being detected after mating. The testes in adult mice are quite small and no mature spermatozoa were detectable in the epididymys or the semineterous tubules. There is a progressive loss of cells in the tubules and some of the tubules had no detectable cells in the adult. In mice, meiosis in males starts at day 14 pp. Prior to the initiation of meiosis, the tubules of MSH5 -/- mice are full of cells and were undistinguishable from +/+ mice. These results show that germ cell migration and the development of testes proceeds normally. The fact that Sertoli cells seem to be unaffected suggests that the intratubular environment is normal.

The failure of spermatagonial maturation is the result of abnormal meiosis. At day 17, wild-type males show all stages of meiosis along with spermatids and spermatozoa. In MSH5 -/- mice, however, only cells in leptolene-zygotene stages of meiosis I were detectable. Although occasional partially paired chromosomes were

detectable, there was a profound lack of chromosome pairing. What is remarkable is that the unpaired chromosomes were found to be associated with COR1 and SYN1, two proteins that are known to be components of synaptonemal complex, and Rad51, a homolog of the bacterial strand transfer protein RecA. In the absence of significant chromosome pairing, the meiotic cells do not appear to progress through meiosis but rather apotose. This meiotic phenotype of mice lacking MSH5 is different from that suggested by studies of MSH4 and MSH5 in S. cerevisiae. MSH4 and MSH5 function as a complex in meiosis and mutations in MSH4 and MSH5 cause a reduction in crossing over but not gene conversion during meiotic recombination. **Sporulation in these mutants occurs with reduced spore viability** which seems to result from chromosome non-disjunction due to the reduced levels of crossing over **(Ross-MacDonald et al, 1994**; Hollingsworth et al., 1995). The sporulation that occurs at reduced levels of crossing over that occurs in S. cerevisiae MSH5 mutants is in contrast to the lack of meiotic progression and apotosis that is observed in conjunction with greatly reduced meiotic chromosome pairing seen in MSH5 mutant male mice.

Crossing over is required for accurate chromosome seggregation during meiosis. Meiotic recombination appears to initiate due to the programed formation of double strand breaks in DNA. These double-strand-breaks have generally been thought to be repaired by classical double-strand-break repair mechanisms resulting in both gene conversion and crossing over **(Szostak et al, 1983)**. It has been suggested that MSH4 and MSH5 are required for correct processing of Holliday junctions during such double-strand-break repair to yield cross overs consistent with the reduction of crossing

over but not gene conversion in MSH4 and MSH5 mutants. Recently a variation of double-strand-break repair models of recombination calles synthesis dependent annealing recombination has proposed to be more consistent with the properties of meiotic recombination than classical double-strand-break repair **(Nassif et al, 1994; Paques et al, 1998)**. In this model, the 3' single stranded end present at one end of a double-strand-break invades the intact homologue and primes DNA synthesis thus copying information from the intact molecule onto one end of the broken molecule. This structure can them be processed in two ways: unwinding and annealing with the other broken end resulting in gene conversion and no crossing over; or conversion to the classical type of double-strand-break repair intermediate containing a double Holliday junction **allowing** both gene conversion and crossing over. If MSH4 and MSH5 were required for the formation of Holliday junctions during synthesis dependent annealing, a defect in this process would yield the lack of extensive chromosome pairing seen in MSH5 mutant mice and the reduced crossing over seem in S. cerevisiae MSH4 and MSH5 mutants. Such a defect in meiosis might lead to apotosis in mammalian cells and partial meiotic arrest, reduced sporulation and reduced spore viability in S. cerevisiae since S. cerevisiae lack apotosis.

Male mice which are deficient in PMS2 and MLH1 are also sterile like the MSH5 deficient mice but the phenotypes observed in each of these three mutant mice is different. Although MLH1 and PMS2 are members of a dimeric complexe that functions in somatic cells, PMS2 -/- mice show abnormal chromosome pairing and spermatogenesis; spermatids as well as spermatozoa, albeit abnormal in appearance,

were detected (Baker et al., 1995). In MLH1 mutant mice, the chromosomes pair but fail to proceed beyond pachytene (Edelmann et al., 1996; Baker et al., 1996). The homology relationships between MSH5 and the MSH proteins that interact with the MLH1-PMS2 heterodimer during mismatch repair suggests the possibility that MSH5 might interact with a MLH1 containing complex in meiosis (Edelmann et al., 1996). However, the different stages of meiosis where abnormalities are detected in MLH1, PMS2 and MSH5 mutant mice **suggest** that the role of these proteins in meiosis may be more complex that the simple model of a MSH4-MSH5 complex and a MLH1-PMS2 complex interacting with each other at the same stage in meiosis. For example the greater severity of the MLH1 mutant phenotype compared to the PMS2 mutant phenotype could reflect the possibility that PMS2 is partially redundant with regards to another MutL related protein such as the recently observed partial redundance between S. cerevisiae PMS1 (PMS2 in humans and mice) and MLH3 **(Flores-Rozas and Kolodner, 1998)**. In addition, the difference in the chromosome pairing defects in the MLH1 and MSH5 mutant mice suggests that these proteins may act at different stages of the homologous pairing process during meiotic recombination.

MSH5 -/- females exhibited the most dramatic phenotype. They were not only sterile but had a progressive degeneration of the ovaries. The lack of ovaries in the adult mice undoubtedly results in hormonal imbalance. The somewhat smaller size of the females that we observed could be attributed to this hormonal imbalance. Although we do not have direct evidence, the failure of the oocytes to proceed through meiosis I suggests that it is due to the lack of pairing of meiotic chromosomes. In normal mice,

pairing of homologous chromosomes is initiated, but the oocytes are arrested at this stage and hormonal stimulation results in the resumption of meiosis. Our results show that the absence of pairing has very profound effects on oocyte development. Oocytes seem to have a checkpoint control prior to the meiotic arrest. In the absence of pairing, the oocytes degenerate. The rapid loss of all oocytes and the attrition of the ovary suggest that pairing is a prerequisite for normal development and maturation of oocytes.

Meiotic abnormalities in the mutant mice. Both male and female msh5-/- mice are infertile as a direct result of a failure in gametogenesis. Males appear normal and exhibit normal reproductive behavior in the presence of wild type females and vaginal plugs were deteced after mating. The sterility in the males is due to the complete absence of spermatozoa either withing the seminfierous tubules or the epididymides. Developmental and morphological analysis of spermatogenesis in msh5 nullizygous males showed normal germ cell migration to the testis, but from day 14 pp, coincident with the onset of prophase I, there is a progressive loss of cells within the seminiferous tubules from the early spermatocyte stage. Despite this loss, seminferous tubule morphology is grossly maintained, with Sertoli cells present and a basal layer of type A and B spermatogonia. In the adult many sections of tubules are completely devoid of cells and the testis size is approximately 30% of wildtype.

The failure of spermatogonial maturation is the result of disrupted meiosis. At day 17 pp, wild type males exhibit cells at all stages of meiosis I within the seminferous tubules. In msh5 -/- males, however, only cells in leptotene to zygotene stages of

meosis I are detected and normal chromosomal pairing is never observed. During the first spermatogenic cycle, this failure of pairing triggers a checkpoint that results in a wave of apoptosis that removes all post-zygotene cells. With each subsequent cycle, the apoptosis continues, resulting in fewer and fewer cells within the tubules and the small adult testis size. Although most chromosomes do not pair, a very small proportion (less than 0.5%) show some degree of synapsis over short lengths of the chromosome though not necessarily involving homologous interactions. Interestingly, the localization of SYCP1 and SYCP3 on the unsynapsed chromosomes suggests that axial elements are constructed in the absence of Msh5 but that the tripartite synaptonemal complex, consisting of 2 homologous axial element-associated chromosomes linked together by a central core element, does not form.

Little is known about the onset of pairing and recombination and their relationship in mammals. In *S. cerevisiae*, the onset of recombination involves the formation of recombination nodules and double strand breaks (dsb), triggered by a complex of proteins including Spo11, Mre11 and Rad50 (Nairz and Klein, 1997), heteroduplex formation and strand exchange. These events preceed, and might be essential for, homologous pairing. In Drosophila, on the other hand, homologous pairing preceeds dsb formation (McKim *et al*, 1998) and other recombination events. In mammals, much of the recent data point towards a mechanism similar to that seen in yeast since proteins known to be involved in dsb formation and repair/stabilization, such as XRCC2, Rad51 and Dmc1 (Cartwright *et al*, 1998; Plug *et* al, 1996; Yoshida *et al*, 1998), are localized in discrete foci (recombination nodules) on unsynapsed meiotic

chromosomes. In msh5 mutant mice, Rad51 is localized on unsynapsed chromosomes **consistent with** the formation of early recombination nodules. This **Rad51 localization** is similar to that seen in Dmc1 mutant mice (Pittman *et al*, 1998), which also block at zygotene (Pittman et al, 1998: Yoshida *et al*, 1998), and suggests that double strand break formation proceeds normally in the absence of **Msh5**. Interestingly, meiotic chromosomes from msh5 -/- spermatocytes also show an increase in Rad51 foci, similar to that seen in dmc1 -/- spermatocytes, compared to that seen on chromosomes from wildtype spermatocytes at zygotene. **However, the lack of extensive chromosome pairing in mice lacking MSH5 suggests** that in the absence of **Msh5** in mice, meiotic crossing over is not initiated and that a complex including **Msh5** is an essential prerequisite for either the initiation and/or maintenance of a synapsed state. **As discussed above, the lack of chromosome pairing in MSH5 mutant mice and the reduced level of crossing over in S. cerevisiae msh5 strains is consistent with the view that Msh5 might act early in recombination to help produce stable, Holliday junction containing recombination intermediates.**

In adult msh5 -/- females, the phenotype is even more dramatic than in males becuase of the complete loss of ovarian structures. Consequently the females do not cycle and are smaller in size, probably as a result of a failure of ovarian steroidogenesis. The disruption of ovarian morphology is evident as early as day 3 pp, even though ovarian size is normal at this stage. Similar to the msh5 -/- males, the germ cells populate the genital ridge but as they progress through prophase I morphological analysis shows that there is widespread chromosomal disruption and the oocytes never

progress beyond zygotene. Thus, the loss of oocytes appears to be result from the failure of synapsis and the activation of a checkpoint resulting in apoptosis, as seen in msh5 -/- spermatocytes, This results in a progressive loss of oocytes from e18. By day 6 pp, there is an almost complete absence of oocytes and the ovary begins to degenerate such that, in the adult, it is usually entirely absent or consists of a few large cysts. The degenerating oocytes fail to initiate folliculogenesis showing that there must be dialog between the oocyte and the surrounding stroma for this process and, in turn, to maintain ovarian morphology.

The phenotype of the msh5 -/- females is remarkably similar to that seen in Turner's syndrome patients. In those patients with a complete loss of one X chromosome, there is acquisition of germ cells into the genital ridge which is followed by a rapid attrition of primary oocytes during the latter part of intrauterine and early neonatal life. This results in adults that have complete gonadal disgenesis, characterized by ÒstreakÓ gonads. It has been suggested that the cause of this degeneration is due to the loss of oocytes as a consequence of the failure of the single X chromosome to pair at zygotene (Singh and Carr 1966). The similarity between the msh5 phenotype and Turner's syndrome patients suggests that this failure of homologous pairing results in the triggering of an apoptotic checkpoint similar to that seen in the msh5 -/- oocytes, resulting in the complete loss of oocytes and ovaries.

EXPERIMENTAL PROCEDURE

Mouse MSH5 cDNA cloning. A segment of the mouse MSH5 gene was obtained by PCR using BALB/c genomic DNA (Clontech) and primers

GTGCTGTGGAATTCAGGATAC (sense) and CCAGAACTCTCTGGAGAAGC (antisense) based on the human cDNA sequence. PCR reactions (25 µl) contained 25 ng genomic DNA template, 0.2 μM each primer, 100 μM each dNTP, 0.75 U Amplitaq polymerase (Perkin-Elmer), 1.5 mM $MgCl_2$ in 10 mM Tris-HCl (pH 8.3), 50 mM KCl. Cycling parameters were 95¡C 5 min, 80¡C 4 min (1 cycle); 95¡C 30 s, 50¡C 15 s, 72¡C 30 s (35 cycles); with a final 5 min extension at 72¡C. **The resulting 118 bp product was purified by agarose gel electrophoresis using the QIAquick Gel Extraction Kit and sequenced directly using the same primers.** The 5' end of the cDNA was cloned by 5' RACE from mouse testis RNA (Clontech) using the 5' RACE Kit (BRL) with modifications of the manufacturer's recommendations. Gene specific primers used for the 5' RACE procedure were CCAGAACTCTCTGGAGAAGC (reverse transcription primer), TGGAGAAGCTTTAGGCTCTCGTGG (first-round amplification primer), and GCATCTGGCATGAAGTGGATAGTGGAG (nested amplification primer). Five-hundred nanograms of poly $(A)^+$ RNA was reverse transcribed in a 25 μl reaction according to the manufacturer's protocol. First strand cDNA was purified using QIAquick PCR Purification Kit (Qiagen) and eluted in 50 μl HPLC-grade water. Aliquots (10 μl) of purified cDNA were used as template in tailing reactions. Five microliters of each tailing reaction was used as template in 50 μl PCR reactions containing 0.2 μM each primer, 100 μM each dNTP, 1.5 U Amplitaq polymerase (Perkin-Elmer), 1.5 mM $MgCl_2$ in 10 mM Tris-HCl (pH 8.3), 50 mM KCl. Following electrophoresis in 1.5% agarose, 0.5? TBE gels, first-round products were purified using the QIAquick Gel Extraction Kit and eluted in 50 μl HPLC-grade water. Purified first-

round products were diluted 1/100 and 2 μl aliquots were used as template for second-round amplification with nested primers. Cycling parameters for both first-round and nested amplifications were 94¡C 5 min, 80¡C 2 min (1 cycle); 94 ¡C 30 s, 57¡C 30 s, 72¡C 30 s (35 cycles); with a final 7 min extension at 72¡C. Products of nested amplifications were purified, cloned and analyzed as described **for human MSH5 RACE products (Winand et al, 1998)**. The remainder of the mouse MSH5 coding sequence was cloned by RT-PCR using the Advantage cDNA PCR Kit and gene-specific primers CTCCACTATCCACTTCATGCCAGATGC (sense) and GCTGGGGAGGACACTGGAA-GGACTCTCA (antisense, based on human 3' untranslated cDNA sequence). Aliquots (5 μl) of cDNA prepared as described, using the 5' RACE Kit (BRL), were amplified in 50 μl PCR reactions containing 0.2 μM each primer, 200 μM each dNTP, 1 μl 50? Advantage KlenTaq polymerase mix in 40 mM tricine-KOH (pH 9.2), 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 75 μg/ml bovine serum albumin. Cycling parameters were 94¡C 1 min (1 cycle); 94¡C 30 s, 55¡C 30 s, 68¡C 4 min (35 cycles) followed by a final 7 min extension at 68¡C. PCR products were purified, cloned and analyzed as described for RACE products.

The mouse MSH5 genomic locus was cloned from a P1 mouse embryonic stem cell genomic library screened by Genome Systems, Inc. A single round of PCR screening with primers GTGCTGTGGAATTCAGGATAC (sense) and CCAGAACTCTCTGGAGAAGC (antisense) yielded three clones 11051, 11052, and 11053. Methods for the preparation of plasmid DNA and the analysis of the mouse

MSH5 intronic sequences **by direct sequencing of the P1 clones** were as described for the human MSH5 gene **(Winand et al, 1998)**.

Construction of the pMSH5ex16 targeting vectors. A genomic *Msh5* fragment containing exon 16 was obtained by screening a mouse genomic Charon 35, 129/Ola phage library with a 334 bp PstI probe corresponding to the region between nucleotides 1524 and 1858 of the mouse *Msh5* cDNA. A 3.8 kb HindIII fragment containing exon 16 was subcloned into pBluescript SK+/- and the intron-exon structure of part of the gene was determined using sequencing and restriction mapping. A 2.0 kb BglII PGKhygro cassette was cloned into the AatII site at codon 528 in exon 16 using BglII/AatII adaptors. The resulting gene targeting clone was designated pMSH5ex16.

Electroporation of embryonic stem cells. The targeting vector pMSH5ex16 (50ug) was linearized at the single XhoI site and electroporated into 5.0×10^7 WW6 embryonic stem cells (Ioffe et al. 1995) and selected with hygromycin (110ug/ml) as described previously (Sirotkin et al., 1995). Colonies were picked after 10 days and their DNA was screened by PCR using forward primer A 5'-AGCTGGAGAACCTGGACTCTC -3' and reverse primer B 5'-TGGAAGGATTGGAGCTACGG-3'. The reaction was performed in a 50 ul reaction mixture containing 100ng DNA, 5ng/ml of each primer, 1.75 mM $MgCl_2$, 0.3 mM of each dNTP and 2.8 U Expand Long Template enzyme mix.(Boehringer Mannheim). Cycling conditions were: 2 min at 94°C (1 cycle), 10 sec at 94°C, 30 sec at 60°C, 3 min at 68°C (10 cycles), 10 sec at 94°C, 30 sec at 60°C, 3 min (plus 20 sec extension/cycle) at 68°C (30 cycles) and 5 min at 68°C (1 cycle). Positive ES

cell colonies were identified by a 1.5 kb PCR fragment specific for the targeting event. Six positive cell lines MSH5-1, MSH5-33, MSH5-41, MSH5-52, MSH5-58, and MSH5-109 were identified and the correct targeting event was shown by NsiI digestion of high molecular weight DNA and Southern Blot analysis using a 0.8kb EcoRI/HindIII probe directed at the 5' intron region between exons13 and 14 that is not included in the targeting vector.

Generation of MSH5 deficient mice. Chimeric mice were generated by injecting C57Bl/6 blastocysts with 8 to 12 embryonic stem cells derived from the MSH5-1 and MSH5- 52 colonies. The two cell lines gave rise to male chimeric animals which were mated with C57Bl/6 females. Chimeras obtained from both cell lines transmitted the *Msh5* mutation through the germline. F1 heterozygotes were interbred to obtain homozygous *Msh5* mutant animals.

Northern Blot Analysis. Total RNA from testis of 24 day old males was extracted and mRNA was isolated using oligo(dT)-cellulose columns (Stratagene mRNA kit). 4 ug of polyA RNA was separated on 1.0% Agarose Formaldehyde gels and transferred onto Nitrocellulose membrane. The membrane was subsequently hybridized with an Msh5 probe corresponding to exons 3 to 8, a probe spanning the complete mouse Msh4 cDNA and a human _-actin probe using the Strip-EZ kit (Ambion).

Western blot analysis. For Western blot analysis equal amounts of protein from testes extracts of 23 day old males were separated on a 10% SDS-polyacrylamide gel and transferred onto a Immobilon-P (Millipore) membrane. The membrane was blocked in TBS, 0.1% Tween-20, 5% nonfat dry milk, 10% goat serum (Sigma) and incubated with

1:1000 diluted primary anti-MSH5 antibody (Palambo et al., 1995). Bound protein was detected by chemiluminescence using a 1:300000 diluted goat anti-mouse IgG horseradish peroxidase conjugate (Sigma).

Histology. Ovaries from *msh5* +/+ and *msh5* -/- females between e18 and 5 wks postpartum (pp) were removed and fixed in Bouins or 4% buffered formalin for 30-360 minutes before transferring to 70% ethanol. Testes were fixed by transcardiac perfusion of 4% buffered formalin and then overnight in fresh fixative. All tissues were processed for histology by routine methods and were sectioned at 3 or 5 ?m.

Chromosomes. Chromosome spreads were prepared according to the method of Counce and Meyer (1973), with modifications. Spreads were then either silver stained in 50% silver nitrate at 65¡C for 6 hours (for electron microscopy) or subjected to immunofluorescence localization of chromosomally-associated proteins, according to the method of Moens and co-workers (Spyropoulos and Moens, 1994). Primary antibodies used were: (1) combined rabbit anti-syn1 and anti-cor1 antibody which detect the synaptonemal complex proteins, SYCP1 and SYCP3, contained within lateral and axial elements of the synaptonemal complex (1:1000) and (2) rabbit anti-Rad51 (1:500). Secondary antibodies were Fab fractions of either FITC or TRITC labelled anti-rabbit IgG (1:1000). Immunofluorescant images were captured used an Olympus Provis Ax70 epifluorescence microscope connected to a cooled (-40¡C) charge-coupled device (Photometrics CH250) and analysed using Scanalytics Cellscan software (Fairfax, VA).

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Figure Legends

Figure 4. Disruption of spermatogenesis in *msh5* -/- males. (A) mRNA expression of *msh5* in testes from wildtype males between the ages of 8 days and 29 days, and in adult wildtype and *msh5* -/- males. (B,C,D,E) H&E stained sections of adult testis from wildtype (B,D) and *msh5* -/- (C,E) males showing loss of spermatocytes beyond zygonema in msh5-deficient males. Le, Leydig cell; S, Sertoli cell; A, type A spermatogonia; B, type B spermatogonia; PL, pre-Leptotene; L, Leptotene spermatocyte; Z, Zygotene spermatocyte; P, Pachytene spermatocyte; RS, round spermatid; ES, elongated spermatid; Sp, spermatozoa. (F, G) Immunolocalization of germ cells using anti-GCNA1 antibody on sections from wildtype (F) and *msh5* -/- (G) testes from 29 day old males showing abundant spermatocytes, seprmatids and spermatozoa in wildtype testes and and few cells in the msh5-deficient testes. For B and C, scale bar = 100 ?m; for D - G, scale bar = 25 ?m.

Figure 5. Progressive depletion of germ cells in *msh5* -/- males during development. (A,B,E,F,I,J) Germ cell immunolocalization using the anti-GCNA1 antibody of testes from wildtype (C,G,K) and *msh5* -/- (D,H,L) males showing the rapid depletion of germ cells from day 17pp onwards in msh5-deficient mice. (C,D,G,H,K,L) TUNEL staining of testes from wildtype (A,E,I) and *msh5* -/- males (B,F,J) showing continuous apoptosis from day 17pp onwards.

Scale bar = 100 ?m

Figure 6. Disruption of meiosis prior to synapsis in *msh5* -/- spermatocytes. (A,B) silver-stained spermatocytes from wildtype (A) and *msh5* -/- (B) testes showing failure of pairing in the absence of msh5. (C,D) Immunofluorescant localization of SYCP1 and SYCP3 proteins on synaptonemal complexes of wildtype pachytene spermatocytes (C) and on axial elements of unsynapsed leptotene/zygotene spermatocytes from *msh5* -/- testes (D). (E,F) Immunoflourescant localization of Rad51 on leptotene spermatocytes from wildtype (E) and *msh5* -/- (F) males.

Figure 7. Loss of oocytes and subsequent ovarian degeneration in *msh5* -/- females. (A,B) Ovaries from day 3pp wildtype (A) and *msh5* -/- (C) females showing oocytes stained with GCNA1. (C) Entire ovary from a day 25pp msh5 -/- female (H&E staining) containing only 3 follicles and degenerating tissue. (D,E) Ovaries from adult wildtype (D) and msh5 -/- females (H&E stained) showing complete loss of oocytes and ovarian architecture in the absence of msh5. B, ovarian bursa;Ov, oviduct. In all cases, scale bar = 200 ?m. (F) Expression of ZP3 and Actin in ovaries of wildtype and msh5 -/- ovaries on day 25pp and in the adult.

Figure 8. Disruption of oogenesis in *msh5* -/- females leads to a failure of folliculogenesis. (A,B,C,D) Ovaries from e18 wildtype (A,B) and *msh5* -/- (C,D) embryos showing oogonia stained with GCNA1 (A,C) or H&E localization of meiotic chromosome detail (C,D). (E,F,G,H) GCNA1 localization of oocytes in ovaries from day

3pp wildtype (E,F) and *msh5* -/- (G,H) females. Arrowheads indicate pachytene oocytes (punctate red staining of nucleus compared to solid red staining of pre-pachytene oocytes), arrows indicate the appearance of the earliest primordial follicles. (I,J,K,L) GCNA1 localization of oocytes in ovaries from day 6pp wildtype (I,J) and *msh5* -/- (K,L) females. Arrows indicate primordial follicles; o, oocyte.For A,C,E,G,I, and K, scale bar = 100 ?m; for B,D,F,H,J,and L, scale bar = 25 ?m.